

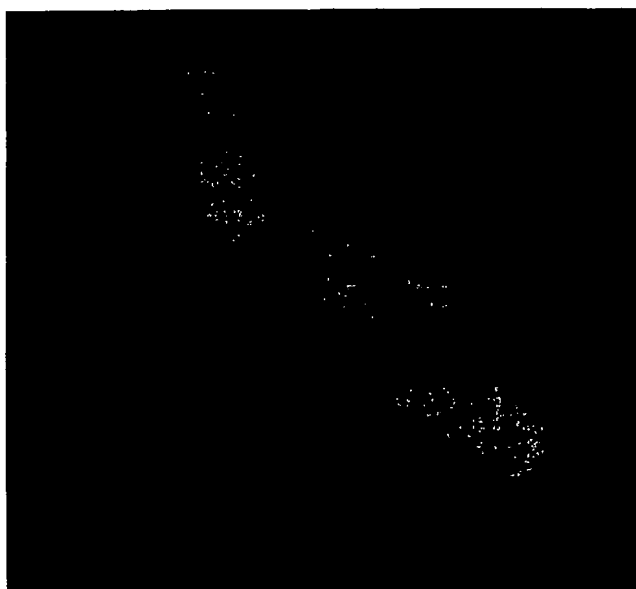
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(54) Title: **ANTIMICROBIAL PEPTIDE, ITS ANALOGS AND ANTIMICROBIAL COMPOSITION COMPRISING THEM**

(57) Abstract: Disclosed is a novel antimicrobial peptide having excellent antimicrobial activities, its analogs and an antimicrobial composition comprising them. The antimicrobial peptide alternatively comprises basic amino acid residues and hydrophobic amino acid residues, and is able to penetrate into microbial cells and act against a wide variety of microorganisms.

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ANTIMICROBIAL PEPTIDE, ITS ANALOGS AND ANTIMICROBIAL COMPOSITION COMPRISING THEM

【Technical field】

5 The present invention relates to antimicrobial peptides. More particularly, the present invention relates to novel peptides exhibiting strong antimicrobial activities against a wide variety of microorganisms including bacteria and fungi; analogs thereof; and antimicrobial composition comprising them.

10

【Background Art】

After discovering a new antimicrobial peptide, cecropin from the silkworm larva as a result of a defense-mechanism in insects against invasion of microorganisms, peptides have begun to be recognized as important
15 biologically active materials. Recent studies show that most of the higher living things accumulate in or secrete into their bodies antimicrobial peptides as a defense-mechanism against pathogens, independently from the immune system. More than 2,000 antimicrobial peptides have been discovered up to date. These peptides found in different species have different amino acid
20 compositions, but the mechanisms of antimicrobial activity are similar to one another.

The most widely known antimicrobial peptides include cecropin, magainin, bombinin, defensin, tachyplesin and buforin. These antimicrobial peptides are composed of 17-24 amino acids, and have antimicrobial activity

against Gram-negative and Gram-positive bacteria as well as protozoa and fungi. Some of these peptides show anti-cancer or anti-viral activity. Especially, magainin is a peptide with 23 amino acids separated from the epidermis of amphibians (Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA*, 84:5449-5453) and can act against human lung cancer cells as well as pathogens. Also, most of the antimicrobial peptides act and kill the target cells specifically and promptly, and exhibit activity spectrum against a wide range of microorganisms (Park, C.B., Kim, M.S. and Kim, S.C. (1996) *Biochem. Biophys. Res. Comm.* 218:408-413).

10 The above antimicrobial peptides

1. have strong antimicrobial activity against a wide variety of microorganisms,
2. are not toxic to human body since they do not destroy host cells, but act specifically against extraneous pathogens,
- 15 3. have little possibility to cause resistance since they show antimicrobial activity by totally different mechanisms from conventional antimicrobial drugs causing resistance,
4. can be mass produced by genetic modification since they do not undergo secondary modification such as glycosylation, and
- 20 5. have high commercial value in pharmaceutical and food industries since they are physico-chemically stable against heat, acid or alkali.

The action mechanism of antimicrobial peptides reported up to now can be categorized into two, as follows;

First, most of the antimicrobial peptides have an action mechanism of destroying membrane potential by increasing cell membrane permeability and stopping the cellular metabolism. Currently, numerous research results are being reported on the biochemical and structural characteristics of the antimicrobial peptides exhibiting the above action mechanism.

Second, a small number of antimicrobial peptides are able to penetrate into microbial cells and strongly act against the microorganisms by combining with DNA or RNA and prohibiting transcription or translation, but the mechanism of this strong antimicrobial activity is not being investigated. However, since antimicrobial drugs with new action mechanism are developed actively due to the emergence of microorganisms that are resistant to antimicrobial drugs, it is important to understand the action mechanism of the antimicrobial peptides that is able to penetrate into microbial cells and act against the microorganisms, and it is also important to develop these antimicrobial peptides.

The salient structural features known to be important in the activity of the antimicrobial peptides that is able to penetrate into microbial cells and act against the microorganisms include,

1. amphipathic helix
 2. distribution of residues stabilizing the above helix,
 3. distribution of basic residues,
 4. distribution of hydrophobic residues,
 5. dipole interaction between charged residues and amphipathic helix,
- and

6. salt-bridge between the residues with different charges.

Noticing the above observations, the present inventors have perfected the present invention by synthesizing new antimicrobial peptides, having amino acid residues of these peptides substituted, added or deleted, and then
5 selecting repeatedly the peptide analogs which is able to penetrate into microbial cells and act against the microorganisms.

Summary of the Invention

The object of the present invention is to provide novel peptides and
10 analogs thereof exhibiting antimicrobial activity against even the microorganisms which are resistant to the traditional antimicrobial peptides, by penetrating into microbial cells and acting against the microorganisms, and an antimicrobial composition comprising them. Also the antimicrobial peptides according to the present invention are novel peptides that have strong
15 antimicrobial activity against a wide variety of microorganisms and negligible or no toxicity when compared to the conventional antimicrobial peptides.

【Detailed Description of the Invention】

20 The present invention relates to novel peptides having antimicrobial activities. More particularly, the present invention relates to novel peptides and analogs thereof exhibiting strong antimicrobial activity against a wide variety of microorganisms including bacteria and fungi, by penetrating into microbial cells and acting against the microorganisms, and antimicrobial

composition comprising them.

The sequence of the amino acids in the present invention was written by using the acronyms according to the nomenclature of IUPAC-IUB.

	alanine	A	arginine	R
5	asparagines	N	aspartic acid	D
	cysteine	C	glutamic acid	E
	glutamine	Q	glycine	G
	histidine	H	isoleucine	I
	leucine	L	lysine	K
10	methionine	M	phenylalanine	F
	proline	P	serine	S
	threonine	T	tryptophane	W
	tyrosine	Y	valine	V

The antimicrobial peptide according to the present invention comprises
15 a central fragment with a relatively conserved amino acid sequence and alternating basic amino acid residues and hydrophobic amino acid residues at the N-terminus and C-terminus sides of the above central fragment. Thereby, the secondary structure of the total peptide is stabilized and the peptide is able to penetrate into microbial cells and act against the microorganisms.

20 The above hydrophobic amino acid can be selected from any hydrophobic amino acids, and preferably from the group consisting of alanine, valine, leucine, and isoleucine. The above basic amino acid can be selected from any basic amino acids, and preferably from the group consisting of lysine, arginine and histidine.

The present invention provides antimicrobial peptide analogs including peptides whose sequence is represented by the following sequence equation

(I);

(I) [N-terminus- $X^1 X^2 X^3 X^4 X^5 X^6 X^7 X^8 X^9 X^{10} X^{11} X^{12} X^{13} X^{14} X^{15}$ - C-terminus]

5

┌ central ┐
fragment

wherein,

X^1 is absent or a basic amino acid;

X^2 are two identical or different hydrophobic amino acids;

10 X^3 is a basic amino acid;

X^4 is glutamine or asparagine;

X^5 is phenylalanine or tryptophane;

X^6 is proline;

X^7 is isoleucine or valine;

15 X^8 is glycine;

X^9 is a basic amino acid;

X^{10} are two identical or different hydrophobic amino acids;

X^{11} are two identical or different basic amino acids;

X^{12} are two identical or different hydrophobic amino acids;

20 X^{13} are two identical or different basic amino acids;

X^{14} are two identical or different hydrophobic amino acids;

X^{15} is absent or a basic amino acid.

The above hydrophobic amino acid can be selected from any hydrophobic amino acids, and preferably from the group consisting of alanine,

25

valine, leucine and isoleucine. The above basic amino acid can be selected from any basic amino acids, and preferably from the group consisting of lysine, arginine and histidine. More preferably, the above antimicrobial peptide can include peptides with amino acid sequences represented by the sequence
 5 identification number (SEQ ID NO) 1 to 34 in the list of sequences in Table 1 and the sequence listing.

Also, the present invention provides antimicrobial peptide analogs including peptides whose sequence is represented by the following sequence equation (II) wherein the residues at N-terminus and C-terminus are
 10 exchanged centering around the central fragment ($X^4 X^5 X^6 X^7 X^8$) of the above sequence equation (I);

(II) [N-terminus- $X^{15} X^{14} X^{13} X^{12} X^{11} X^{10} X^9 X^4 X^5 X^6 X^7 X^8 X^3 X^2 X^1$ -C-terminus]

└ central ┐
fragment

15 wherein,

X^1 is absent or a basic amino acid;

X^2 are two identical or different hydrophobic amino acids;

X^3 is a basic amino acid;

X^4 is glutamine or asparagine;

20 X^5 is phenylalanine or tryptophane;

X^6 is proline;

X^7 is isoleucine or valine;

X^8 is glycine;

X^9 is a basic amino acid;

25 X^{10} are two identical or different hydrophobic amino acids;

X¹¹ are two identical or different basic amino acids;

X¹² are two identical or different hydrophobic amino acids;

X¹³ are two identical or different basic amino acids;

X¹⁴ are two identical or different hydrophobic amino acids;

5 X¹⁵ is absent or a basic amino acid.

The above hydrophobic amino acid can be selected from any hydrophobic amino acids, and preferably from the group consisting of alanine, valine, leucine and isoleucine. The above basic amino acid can be selected
10 from any basic amino acids, and preferably from the group consisting of lysine, arginine and histidine. More preferably, the above antimicrobial peptide can include peptides with amino acid sequences represented by SEQ ID NO: 35 to 68 in the list of sequences in Table 1 and the sequence listing.

Also, the present invention provides antimicrobial peptide analogs
15 including the peptides represented by the above sequence equation (I) and (II), which are amidated at C-terminus. Preferably, the above antimicrobial peptide can include peptides with amino acid sequences represented by SEQ ID NO: 69 to 72 in the list of sequences in Table 1 and the sequence listing. As can be seen in Table 2, the antimicrobial peptides whose C-terminus is amidated
20 show improved antimicrobial activities against Gram-positive and Gram-negative bacteria, and fungi.

Also the present invention provides antimicrobial compositions comprising one or more antimicrobial peptides according to the present invention as effective ingredients in a pharmacologically effective amount.

The above antimicrobial composition can comprise pharmacologically acceptable carriers or other known antimicrobial materials in addition to the antimicrobial peptides according to the present invention.

The peptides according to the present invention can be synthesized by
5 using the methods known to person skilled in the field, for example by using automatic peptide synthesizer. For instance, the above peptide according to the present invention can be obtained by genetic modification technique by synthesizing the gene encoding the fusion protein including the peptide of the present invention by genetic modification, by transforming the host
10 microorganisms with the synthesized gene, and by obtaining the peptide from the fusion protein separated from the host microorganism.

The preparation method of the antimicrobial peptide according to the preferable specific embodiment of the present invention includes the steps of

- synthesizing a peptide by using automatic peptide synthesizer;
- 15 - measuring antimicrobial activity, cell penetration activity and hemolytic activity of the above synthesized peptide;
- synthesizing the peptide analogs wherein the amino acid residues of the above synthesized peptide is substituted, added or deleted; and
- 20 - selecting the peptide analog with strong antimicrobial activity and high safety by repeating the above steps.

The microorganisms used in the present invention include Gram-positive bacteria such as *Bacillus subtilis* (ATCC 62037), *Staphylococcus aureus* (ATCC 15752) and *Streptococcus mutans* (ATCC 25175), Gram-

negative bacteria such as *Escherichia coli* (ATCC 27325), *Salmonella enteritidis* (ATCC 15277) and *Pseudomonas putida* (ATCC 17426) and fungi such as *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 44774) and *Cryptococcus neoformans* (ATCC 34881), obtained from American
5 Type Culture Collection (ATCC).

【Brief Description of Drawings】

Figures 1A ~ 1D are the results of analyzing the cell penetration activity of the
10 new antimicrobial peptides by confocal microscopy.

Examples

This invention is explained in more detail based on the following
Examples but they should not be construed as limiting the scope of this
15 invention.

Example 1

Preparation of new antimicrobial peptide analogs

The peptides with the amino acid sequences listed in the below Table1
20 were synthesized by using automatic peptide synthesizer (Milligen 9050, Millipore, USA) and were separated and purified by using C18 reverse phase High Performance Liquid Chromatography (HPLC, Waters Associates, USA).

TABLE 1: Amino acid sequence of peptide analogs

Peptide	Amino Acid Sequence
SEQ ID NO 1	RVVRQWPIGRVVRRVVRRVVR
SEQ ID NO 2	KVVKQWPIGKVVKKVVKKVVK
SEQ ID NO 3	RLLRQWPIGRLLRLLRLLR
SEQ ID NO 4	KLLKQWPIGKLLKLLKLLK
SEQ ID NO 5	RVLRQWPIGRVLRRVLRRVLR
SEQ ID NO 6	KVLKQWPIGKVLKKVLKKVLK
SEQ ID NO 7	RLVRQWPIGRLVRRLVRRLVR
SEQ ID NO 8	KLVKQWPIGKLVKKLVKKLVK
SEQ ID NO 9	RVVKQWPIGRVVKRVVKRVVK
SEQ ID NO 10	KVVRQWPIGKVVRKVVRKVVR
SEQ ID NO 11	RLLKQWPIGRLLKRLLKRLLK
SEQ ID NO 12	KLLRQWPIGKLLRKLLRKLLR
SEQ ID NO 13	RVLKQWPIGRVLKRVLKRVLK
SEQ ID NO 14	KVLRQWPIGKVLRKVLRKVLR
SEQ ID NO 15	RLVKQWPIGRLVKRLVKRLVK
SEQ ID NO 16	KLVRQWPIGKLVRKLVRKLVR
SEQ ID NO 17	KLVRQFPVGKLVRKLVRKLVR
SEQ ID NO 18	RVVRNWPIGRVVRRVVRRVVR
SEQ ID NO 19	KVVKNWPIGKVVKKVVKKVVK
SEQ ID NO 20	RLLRNWPIGRLLRLLRLLR
SEQ ID NO 21	KLLKNWPIGKLLKLLKLLK
SEQ ID NO 22	RVLRNWPIGRVLRRVLRRVLR
SEQ ID NO 23	KVLKNWPIGKVLKKVLKKVLK
SEQ ID NO 24	RLVRNWPIGRLVRRLVRRLVR
SEQ ID NO 25	KLVKNWPIGKLVKKLVKKLVK
SEQ ID NO 26	RVVKNWPIGRVVKRVVKRVVK
SEQ ID NO 27	KVVRNWPIGKVVRKVVRKVVR
SEQ ID NO 28	RLLKNWPIGRLLKRLLKRLLK
SEQ ID NO 29	KLLRNWPIGKLLRKLLRKLLR
SEQ ID NO 30	RVLKNWPIGRVLKRVLKRVLK
SEQ ID NO 31	KVLRNWPIGKVLRKVLRKVLR
SEQ ID NO 32	RLVKNWPIGRLVKRLVKRLVK
SEQ ID NO 33	KLVRNWPIGKLVRKLVRKLVR
SEQ ID NO 34	KLVRNFPVGKLVRKLVRKLVR
SEQ ID NO 35	RVVRRVVRRVVRQWPIGRVVR
SEQ ID NO 36	KVVKKVVKKVVKQWPIGKVVK
SEQ ID NO 37	RLLRLLRLLRQWPIGRLLR
SEQ ID NO 38	KLLKLLKLLKQWPIGKLLK
SEQ ID NO 39	RVLRRVLRRVLRQWPIGRVLR
SEQ ID NO 40	KVLKKVLKKVLKQWPIGKVLK

SEQ ID NO 41	RLVRRRLVRRRLVRQWPIGR LVR
SEQ ID NO 42	KLVKKLVKKLVKQWPIGKLVK
SEQ ID NO 43	RVVKRVVKRVVKQWPIGRVVK
SEQ ID NO 44	KVVRKVVRKVVRQWPIGKVVR
SEQ ID NO 45	RLLKRLLKRLLKQWPIGRLLK
SEQ ID NO 46	KLLRKLLRKLLRQWPIGKLLR
SEQ ID NO 47	RVLKRVLKRVLKQWPIGRVLK
SEQ ID NO 48	KVLRKVLRKVLRQWPIGKVLR
SEQ ID NO 49	RLVKRLVKRLVKQWPIGRLVK
SEQ ID NO 50	KLVRKLVRKLVRQWPIGKLVR
SEQ ID NO 51	KLVRKLVRKLVRQFPVGKLVR
SEQ ID NO 52	RVVRRVVRVVRNWPIGRVVR
SEQ ID NO 53	KVVKKVVKVVKVKNWPIGKVVK
SEQ ID NO 54	RLLRLLRLLRNWPIGRLLR
SEQ ID NO 55	KLLKKLLKLLKNWPIGKLLK
SEQ ID NO 56	RVLRRVLRVLRNWPIGRVLR
SEQ ID NO 57	KVLKKVLKKVLKNWPIGKVLR
SEQ ID NO 58	RLVRRLVRRLVNRNWPIGR LVR
SEQ ID NO 59	KLVKKLVKKLVKNWPIGKLVK
SEQ ID NO 60	RVVKRVVKRVVKVKNWPIGRVVK
SEQ ID NO 61	KVVRKVVRKVVRNWPIGKVVR
SEQ ID NO 62	RLLKRLLKRLLKNWPIGRLLK
SEQ ID NO 63	KLLRKLLRKLLRNWPIGKLLR
SEQ ID NO 64	RVLKRVLKRVLKNWPIGRVLK
SEQ ID NO 65	KVLRKVLRKVLRNWPIGKVLR
SEQ ID NO 66	RLVKRLVKRLVKVKNWPIGR LVK
SEQ ID NO 67	KLVRKLVRKLVRNWPIGKLVR
SEQ ID NO 68	KLVRKLVRKLVRNFPVGKLVR
SEQ ID NO 69	KLVRQWPIGKLVRKLVRKLVR-amide
SEQ ID NO 70	RLVKVKNWPIGR LVKRLVKRLVK-amide
SEQ ID NO 71	KVLRKVLRKVLRQWPIGKVLR-amide
SEQ ID NO 72	RVLKRVLKRVLKNWPIGRVLK-amide

Example 2

Determination of antimicrobial activity of peptides and their analogs

The antimicrobial activity of the peptides prepared in Example 1 was
5 determined against microorganisms by 96-well microdilution minimal inhibitory
concentration assay. After overnight culturing the bacteria and fungi in

trypticase soy broth (TSB) and Saboraud (SAB) at 37 °C and 30 °C, respectively, they were inoculated in new media and cultured for 2 hours to exponential growth phase. After diluting the microorganisms to 10^5 per 1 ml, 190 μ l was inoculated in each 96-well plate, and 10 μ l of serially diluted peptides were added in each well. The 96-well plate was cultured for 12 hours, and the absorbance was determined in well to determine the minimum concentration where the microorganisms cannot grow as the minimum inhibitory concentration (MIC). The result is shown in Table 2.

As can be seen in Table 2, MIC of the peptides prepared in Example 1 was 1-2 μ l, whereas MIC of magainin was 32-128 μ l against Gram-positive bacteria, Gram-negative bacteria and fungi. The results indicate that the peptides prepared in Example 1 have 32-128 times stronger antimicrobial activity than magainin.

TABLE 2: Minimum Inhibitory Concentration of Peptide analogs

Microorganism	Minimum Inhibitory Concentration (μ g/ml)										
	SEQ ID NO										
	1	2	3	4	5	8	10	11	13	16	17
Gram-positive bacteria											
<i>Bacillus subtilis</i>	2	1	2	2	1	1	1	1	2	2	2
<i>Staphylococcus aureus</i>	1	1	1	1	1	1	1	1	1	1	1
<i>Streptococcus mutans</i>	2	2	1	1	1	2	2	2	2	2	2
Gram-negative bacteria											
<i>Escherichia coli</i>	1	1	2	1	1	1	1	1	1	1	1
<i>Salmonella enteritidis</i>	1	1	1	1	1	1	1	2	1	1	1
<i>Pseudomonas putida</i>	1	1	1	1	1	1	1	1	1	1	1
Fungi											
<i>Candida albicans</i>	2	1	1	1	1	2	2	1	1	1	1
<i>Cryptococcus neoformans</i>	1	1	1	1	1	1	1	1	1	1	1
<i>Saccharomyces cerevisiae</i>	2	2	2	2	2	2	2	2	2	1	1

Microorganism	Minimum Inhibitory Concentration (µg/ml)											
	SEQ ID NO											
	18	19	20	21	23	24	26	29	31	32	34	
Gram-positive bacteria												
<i>Bacillus subtilis</i>	1	2	2	1	1	1	1	1	1	1	1	
<i>Staphylococcus aureus</i>	1	1	1	1	1	1	1	1	1	1	1	
<i>Streptococcus mutans</i>	2	1	2	2	2	1	1	2	2	2	2	
Gram-negative bacteria												
<i>Escherichia coli</i>	1	2	1	1	1	1	2	1	1	1	1	
<i>Salmonella enteritidis</i>	2	1	2	2	2	2	1	1	1	1	1	
<i>Pseudomonas putida</i>	1	1	1	1	1	1	1	1	1	1	1	
Fungi												
<i>Candida albicans</i>	1	1	1	2	1	2	2	1	1	1	1	
<i>Cryptococcus neoformans</i>	1	1	1	1	1	1	1	1	1	1	1	
<i>Saccharomyces cerevisiae</i>	1	2	2	1	1	2	2	2	2	2	1	

Microorganism	Minimum Inhibitory Concentration (µg/ml)										
	SEQ ID NO										
	35	36	37	38	39	42	44	45	47	50	51
Gram-positive bacteria											
<i>Bacillus subtilis</i>	1	1	1	1	2	1	2	1	1	1	1
<i>Staphylococcus aureus</i>	1	1	1	1	2	1	1	1	1	1	1
<i>Streptococcus mutans</i>	1	2	1	2	2	2	2	1	2	1	1
Gram-negative bacteria											
<i>Escherichia coli</i>	2	2	1	2	1	1	1	1	1	1	1
<i>Salmonella enteritidis</i>	1	1	2	1	1	1	1	2	1	2	2
<i>Pseudomonas putida</i>	1	1	1	1	1	1	1	1	1	1	1
Fungi											
<i>Candida albicans</i>	1	2	2	2	2	1	2	2	1	1	1
<i>Cryptococcus neoformans</i>	1	1	2	1	1	1	1	2	1	1	1
<i>Saccharomyces cerevisiae</i>	2	2	2	2	2	1	1	2	2	2	2

Microorganism	Minimum Inhibitory Concentration (µg/ml)											
	SEQ ID NO											
	52	53	54	55	57	58	60	63	65	66	68	
Gram-positive bacteria												
<i>Bacillus subtilis</i>	1	1	1	1	1	1	1	1	2	1	1	
<i>Staphylococcus aureus</i>	1	1	1	1	1	1	2	1	1	1	1	
<i>Streptococcus mutans</i>	2	1	2	2	2	1	2	2	2	2	2	
Gram-negative bacteria												
<i>Escherichia coli</i>	1	1	2	2	2	1	2	1	1	2	1	
<i>Salmonella enteritidis</i>	2	1	1	1	1	1	2	2	2	1	1	
<i>Pseudomonas putida</i>	1	1	1	1	1	1	1	1	1	1	1	
Fungi												
<i>Candida albicans</i>	1	2	1	1	1	2	1	1	1	1	1	
<i>Cryptococcus neoformans</i>	1	1	1	1	1	1	1	1	1	1	1	
<i>Saccharomyces cerevisiae</i>	1	1	2	2	2	2	1	2	1	2	2	

Microorganism	Minimum Inhibitory Concentration (µg/ml)				
	SEQ ID NO				
	69	70	71	72	Magainin
Gram-positive bacteria					
<i>Bacillus subtilis</i>	1	1	1	1	64
<i>Staphylococcus aureus</i>	1	1	1	1	64
<i>Streptococcus mutans</i>	1	2	1	2	128
Gram-negative bacteria					
<i>Escherichia coli</i>	1	1	1	1	128
<i>Salmonella enteritidis</i>	2	1	1	1	32
<i>Pseudomonas putida</i>	1	1	1	1	64
Fungi					
<i>Candida albicans</i>	1	1	1	1	32
<i>Cryptococcus neoformans</i>	1	1	1	1	32
<i>Saccharomyces cerevisiae</i>	2	1	2	2	32

Example 3

Determination of cell penetration activity of peptides and their analogs

The cell penetration activity of the peptides prepared in Example 1 was observed by confocal microscopy. After inoculating and culturing *E. coli* in trypticase soy broth at 37 °C overnight, they were inoculated in new media and cultured for 2 hours to exponential growth phase. After washing *E. coli* with 10 mM NAPB (sodium phosphate buffer) and diluting to 10⁵ CFU/ ml, the above diluted *E. coli* was fixed for 30 min on glass-slides coated with poly-L-lysine. The N-terminus of the peptides prepared in Example 1 was labeled with FITC (fluorescein isothiocyanate), and the labeled peptides were applied to *E. coli* fixed on the glass-slides for 5 minutes. The glass-slides were washed with 10 mM NAPB and observed them by confocal microscopy. The obtained results are shown in Figure 1a ~ 1d.

Figures 1A, 1B and 1C are photographs obtained by confocal microscopy showing that peptides of SEQ ID NO: 1, 33 and 65 penetrate into

E. coli cells. Figure 1D is a photograph obtained by confocal microscopy of *E. coli* treated with magainin, which can bind with cell membrane to kill the microorganisms.

5 Example 4

Measurement of hemolytic activity of peptides and their analogs

 After separating the precipitated human red blood cells (hRBC) from 3 ml of human blood, they were washed with PBS (phosphate buffered saline) and diluted to make the total volume of 20 ml. In 190 μ l of the prepared
10 hRBC solution, 10 μ l of each peptide sample (4 μ g/ μ l) prepared in Example 1 was added to make the final concentration of 200 μ g/ml, reacted for 1 h at 37 °C and centrifuged for 5 min at 4000 rpm. After diluting 100 μ l of the supernatant of each sample by 10 times in PBS buffer solution, absorbance was measured at 576 nm (A_{576}). The absorbance of the sample treated with
15 0.2 % Triton X-100 was set to represent 100 % hemolysis and the percent (%) hemolysis of each sample was relatively calculated from each measured absorbance, as shown in Table 3.

 As can be seen in Table 3, all of the peptides prepared in Example 1 showed less than 1 % of hemolysis activities. Such result implies that the
20 peptides prepared in Example 1 are not toxic to human cells. In contrast, melittin, which was included in this Example for comparison, is a hemolytic peptide and did destroy almost all hRBC at the concentration of 200 μ g/ml.

Table 3: Hemolytic activity of peptide analogs

SEQ ID NO	A567	% hemolysis	SEQ ID NO	A567	% hemolysis
1	0.013	0.4	2	0.017	0.5
3	0.022	0.6	4	0.025	0.8
5	0.019	0.6	8	0.021	0.7
10	0.016	0.5	11	0.013	0.4
13	0.011	0.3	16	0.014	0.4
17	0.012	0.4	18	0.014	0.4
19	0.015	0.5	20	0.018	0.6
21	0.012	0.4	23	0.013	0.4
24	0.016	0.5	26	0.011	0.3
29	0.015	0.5	31	0.012	0.4
32	0.013	0.4	34	0.011	0.3
35	0.017	0.5	36	0.018	0.6
37	0.016	0.5	38	0.019	0.6
39	0.022	0.6	42	0.014	0.4
44	0.021	0.7	45	0.015	0.5
47	0.017	0.5	50	0.013	0.4
51	0.016	0.5	52	0.016	0.5
53	0.015	0.5	54	0.010	0.3
55	0.020	0.6	57	0.025	0.8
58	0.013	0.4	60	0.016	0.5
63	0.012	0.4	65	0.023	0.7
66	0.015	0.5	68	0.015	0.5
69	0.018	0.6	70	0.017	0.5
71	0.013	0.4	72	0.015	0.5
0.2% Triton X-100	3.21	100	Melittin	3.17	99

【Industrial Applicability】

As written above, the antimicrobial peptides and their analogs
 5 synthesized in the present invention show strong antimicrobial activity against
 Gram-positive bacteria, Gram-negative bacteria and fungi. Since the peptides
 of the present invention strongly inhibits the growth of microorganisms without
 hemolytic activity, the peptides of the present invention can be used as
 excellent antimicrobial agents such as wound healing enhancer, external

wound treatment agent, mouth wash, eye-drops, etc. Therefore the present invention will become valuable in the biomedical industry.

CLAIMS

1. An antimicrobial peptide including the amino acid sequence represented as the following sequence equation (I):

5 N-terminus - X¹X²X³X⁴X⁵X⁶X⁷X⁸X⁹X¹⁰X¹¹X¹²X¹³X¹⁴X¹⁵ - C-terminus - (I)

wherein,

X¹ is absent or a basic amino acid;

X² are two identical or different hydrophobic amino acids;

X³ is a basic amino acid;

10 X⁴ is glutamine or asparagine;

X⁵ is phenylalanine or tryptophane;

X⁶ is proline;

X⁷ is isoleucine or valine;

X⁸ is glycine;

15 X⁹ is a basic amino acid;

X¹⁰ are two identical or different hydrophobic amino acids;

X¹¹ are two identical or different basic amino acids;

X¹² are two identical or different hydrophobic amino acids;

X¹³ are two identical or different basic amino acids;

20 X¹⁴ are two identical or different hydrophobic amino acids;

X¹⁵ is absent or a basic amino acid.

2. The antimicrobial peptide according to claim 1 including one of the amino acid sequences selected from SEQ ID NO: 1 to 34.

3. An antimicrobial peptide including the amino acid sequence represented as the following sequence equation (II):

N-terminus - $X^{15}X^{14}X^{13}X^{12}X^{11}X^{10}X^9X^4X^5X^6X^7X^8X^3X^2X^1$ -C-terminus - (II)

wherein,

- 5 X^1 is absent or a basic amino acid;
- X^2 are two identical or different hydrophobic amino acids;
- X^3 is a basic amino acid;
- X^4 is glutamine or asparagine;
- X^5 is phenylalanine or tryptophane;
- 10 X^6 is proline;
- X^7 is isoleucine or valine;
- X^8 is glycine;
- X^9 is a basic amino acid;
- X^{10} are two identical or different hydrophobic amino acids;
- 15 X^{11} are two identical or different basic amino acids;
- X^{12} are two identical or different hydrophobic amino acids;
- X^{13} are two identical or different basic amino acids;
- X^{14} are two identical or different hydrophobic amino acids;
- X^{15} is absent or a basic amino acid.

20

4. The antimicrobial peptide according to claim 3 including one of the amino acid sequences selected from SEQ ID NO: 33 to 68.

5. The antimicrobial peptide according to claim 1, wherein the C-

terminus of the amino acid sequence is amidated.

6. The antimicrobial peptide according to claim 2, wherein the C-terminus of the amino acid sequence is amidated.

5

7. The antimicrobial peptide according to claim 3, wherein the C-terminus of the amino acid sequence is amidated.

8. The antimicrobial peptide according to claim 4, wherein the C-terminus of the amino acid sequence is amidated.

10

9. An antimicrobial peptide including one of the amino acid sequences selected from SEQ ID NO: 69 to 72.

15

10. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 1 as effective ingredients in a pharmacologically effective amount.

20

11. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 2 as effective ingredients in a pharmacologically effective amount.

12. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 3 as effective ingredients in a pharmacologically

effective amount.

13. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 4 as effective ingredients in a pharmacologically
5 effective amount.

14. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 5 as effective ingredients in a pharmacologically effective amount..
10

15. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 6 as effective ingredients in a pharmacologically effective amount.

16. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 7 as effective ingredients in a pharmacologically effective amount.
15

17. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 8 as effective ingredients in a pharmacologically effective amount.
20

18. An antimicrobial composition comprising one or more antimicrobial peptides according to claim 9 as effective ingredients in a pharmacologically

effective amount.

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FIG. 1A

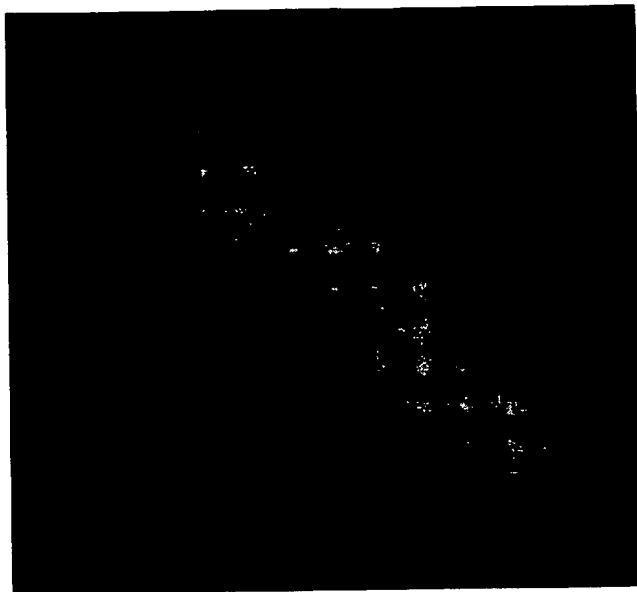
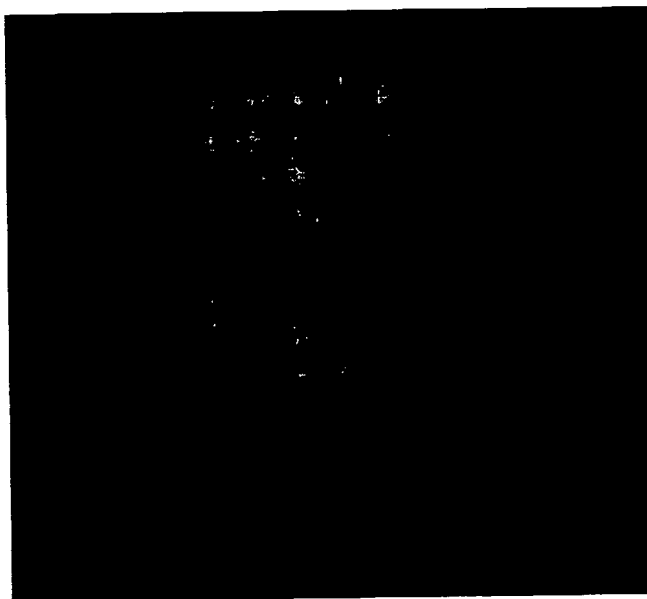


FIG. 1B



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FIG. 1C

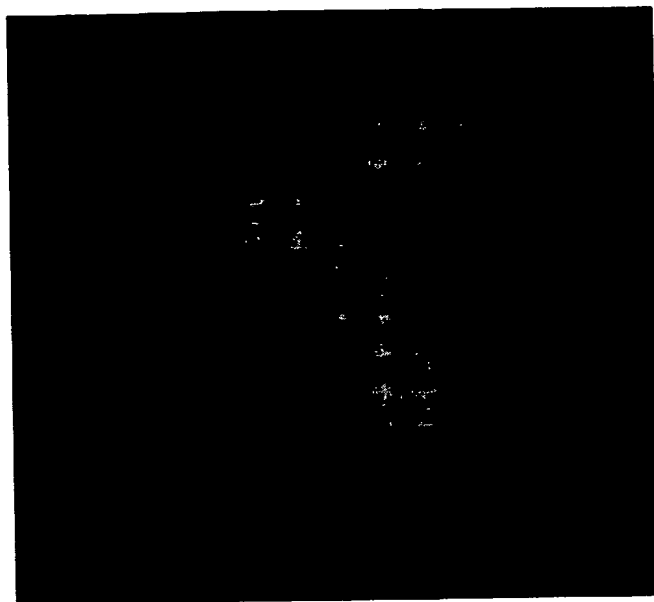
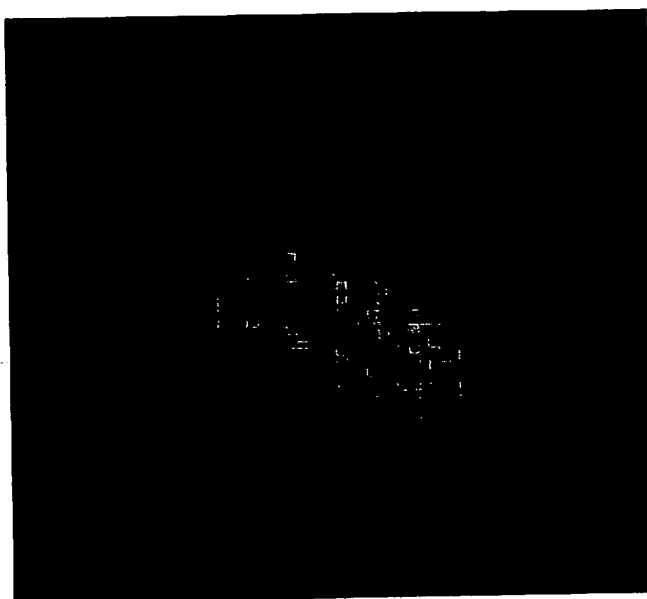


FIG. 1D



【Sequence Listing】

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR03/00602

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C07K 7/08**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 07/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI GenBank, CA, USPTO, Espacenet, PAJ, "antimicrobial", "peptide", "synthesize"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,830,993A(KANSAS STATE UNIVERSITY RESEARCH FOUNDATION) 03. 11. 1998. see the whole documents	1 - 18
A	US 5,607,914A(PIONEER HI-BRED INTERNATIONAL INC.) 03. 11. 1998. see the whole documents	1 - 18
A	JP 9-165342A(MORINAGA MILK IND CO LTD.) 24. 06. 1997. see the whole documents	1 - 18
A	JP 8-143596A(AMANO PHARMACEUT CO LTD.) 04. 06. 1996. see the whole documents	1 - 18
A	WO 99/26971A1(THE UNIVERSITY OF MELBOURNE, VICTORIAN DAIRY IND) 03. 06. 1999, see the whole documents	1 - 18

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 JULY 2003 (10.07.2003)

Date of mailing of the international search report

11 JULY 2003 (11.07.2003)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR03/00602

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US5830993A	03.11.1998	US5830993A W09632129A1	03.11.1998 17.10.1996
US5607914A	04.03.1997	US5607914A	04.03.1997
JP9-165342A	24.06.1997	none	
JP8-143596A	04.06.1996.	none	
W09926971A1	03.06.1999	EP1032592A1 W09926971A1 ZA9810679A	06.09.2000 03.06.1999 30.07.1999